Unravelling Stimulus Direction Dependency of Visual Acuity in Larval Zebrafish by Consistent Eye Displacements Upon Optokinetic Stimulation

Bögli, S Y ; Afthinos, M ; Bertolini, G ; Straumann, D ; Huang, M Y Y

Abstract: PURPOSE Impairment of visual acuity (VA) can be seen early on in various diseases and has a major impact on patients' daily activities. Zebrafish is an important model for studying visual disorders. We developed a new method in zebrafish larva to easily and precisely measure the VA, which should allow for better estimation of affected vision such as after genetic manipulation or pharmacologic intervention. METHODS We used an optokinetic reflex (OKR) paradigm with a staircase technique to estimate VA of zebrafish larva. Consistent eye displacements were used as the indicator for OKR. We measured VA and determined the dependence of VA on clockwise and counterclockwise horizontal stimulus directions. RESULTS Visual acuity in zebrafish larva was estimated to be 0.179 ± 0.013 cyc/deg binocularly and 0.129 ± 0.008 cyc/deg (left eye) and 0.128 ± 0.012 cyc/deg (right eye) monocularly. We found within single subjects spatial frequency thresholds that showed highly significant differences between the two horizontal stimulus directions. Average higher and lower binocular thresholds were 0.181 ± 0.026 and 0.158 ± 0.014 cyc/deg, respectively. Importantly, no correlations were found between spatial frequency thresholds and average median peak slow-phase eye velocities (SPV) of OKR in all experiments. CONCLUSIONS Consistent eye displacements evoked by OKR stimuli can be used as an indirect measure of VA in zebrafish larva. Conversely, using SPV of OKR to determine VA does not seem to be accurate. With our method, single larva showed significantly different VA depending on stimulus directions, which might reflect asymmetric maturation of retinal and/or visual pathway structures.

DOI: https://doi.org/10.1167/iovs.15-18072

Originally published at:
Bögli, S Y; Afthinos, M; Bertolini, G; Straumann, D; Huang, M Y Y (2016). Unravelling Stimulus Direction Dependency of Visual Acuity in Larval Zebrafish by Consistent Eye Displacements Upon Optokinetic Stimulation. Investigative Ophthalmology Visual Science [IOVS], 57(4):1721-1727.
DOI: https://doi.org/10.1167/iovs.15-18072

Unravelling Stimulus Direction Dependency of Visual Acuity in Larval Zebrafish by Consistent Eye Displacements Upon Optokinetic Stimulation

Stefan Yu Bögli,[1] Maresa Afthinos,[1] Giovanni Bertolini,[1] Dominik Straumann,[1–3] and Melody Ying-Yu Huang[1–3]

[1]Department of Neurology, University Hospital Zurich, University of Zurich, Switzerland
[2]Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Switzerland
[3]Neuroscience Center Zurich (ZNZ), University of Zurich and ETH Zurich, Switzerland

Correspondence: Melody Ying-Yu Huang, Department of Neurology, University Hospital Zurich, University of Zurich, Frauenklinikstrasse 26, CH-8091, Zürich, Switzerland; Ying-Yu.Huang@usz.ch.

Submitted: August 29, 2015
Accepted: February 26, 2016

Citation: Bögli SY, Afthinos M, Bertolini G, Straumann D, Huang MY-Y. Unravelling stimulus direction dependency of visual acuity in larval zebrafish by consistent eye displacements upon optokinetic stimulation. *Invest Ophthalmol Vis Sci*. 2016;57:1721–1727. DOI:10.1167/iovs.15-18072

Purpose. Impairment of visual acuity (VA) can be seen early on in various diseases and has a major impact on patients' daily activities. Zebrafish is an important model for studying visual disorders. We developed a new method in zebrafish larva to easily and precisely measure the VA, which should allow for better estimation of affected vision such as after genetic manipulation or pharmacologic intervention.

Methods. We used an optokinetic reflex (OKR) paradigm with a staircase technique to estimate VA of zebrafish larva. Consistent eye displacements were used as the indicator for OKR. We measured VA and determined the dependence of VA on clockwise and counterclockwise horizontal stimulus directions.

Results. Visual acuity in zebrafish larva was estimated to be 0.179 ± 0.013 cyc/deg binocularly and 0.129 ± 0.008 cyc/deg (left eye) and 0.128 ± 0.012 cyc/deg (right eye) monocularly. We found within single subjects spatial frequency thresholds that showed highly significant differences between the two horizontal stimulus directions. Average higher and lower binocular thresholds were 0.181 ± 0.026 and 0.158 ± 0.014 cyc/deg, respectively. Importantly, no correlations were found between spatial frequency thresholds and average median peak slow-phase eye velocities (SPV) of OKR in all experiments.

Conclusions. Consistent eye displacements evoked by OKR stimuli can be used as an indirect measure of VA in zebrafish larva. Conversely, using SPV of OKR to determine VA does not seem to be accurate. With our method, single larva showed significantly different VA depending on stimulus directions, which might reflect asymmetric maturation of retinal and/or visual pathway structures.

Keywords: visual acuity, zebrafish, optokinetic nystagmus

Visual acuity (VA) may be thought of as how well the eye is able to see fine detail, which describes acuteness, sharpness, or clearness of the vision. It measures the spatial resolving power of the visual system, particularly with its ability to distinguish letters or numbers at a given distance. Clinically it describes the minimal angle in which two visual objects can still be perceived separately.[1–3] In human beings, VA does not only depend on optical factors that are related to the eye such as the cornea or the lense, but also involves higher cognitive functions. Hence, degraded VA can be associated with various neurological disorders such as optic neuritis,[4] commonly caused by multiple sclerosis, and stroke[5] that causes damage to optic fibers transmitting visual information from the eyes to the occipital lobe. Clinically, VA is measured by the widely used Snellen chart or other optotype charts such as Landolt C, E charts, or logMAR chart.[1–3] In recent years, gratings with alternating black and white stripes have also been used to analyze vision,[2,6,7] Measuring VA with gratings is simpler compared with using an eye chart because subjects only have to detect the grating without having to identify any symbol. Gratings can be characterized by their spatial frequency, contrast and type, such as sine- or square-wave grating patterns. Hereupon, VA is estimated by the highest spatial frequency, defined as threshold frequency, at which the grating can still be perceived. Usually humans have a threshold frequency of 30 to 40 cyc/deg.[3] Visual acuity assessment with gratings can be especially practical in infants or patients who cannot verbally respond. Infants show a behavior called preferential looking, which refers to the preference in children of rather looking at a grating than a plain surface.[8,9] The examiner observes the eye movements of the infant while showing it gratings with different spatial frequencies next to a blank space. This test, however, is rather time consuming. In order to simplify the method, the optokinetic reflex (OKR) can instead be used to assess VA in infants in a similar manner.[9,10]

Optokinetic reflex is a reflexive eye movement caused by whole-field movements of the visual scene. The response consists of smooth tracking of the moving gratings across the visual field followed by fast saccadic movements in the opposite direction to reset the eyes.[8–11] Such stereotyped OKR eye movements are robust and well conserved among species, and therefore offer a reliable measurement of basic

visual functioning without requirement of any prior training or vocal responses. Infants are placed in a bed facing a round screen. By presenting them moving gratings the OKR can be evoked and measured. The spatial frequency threshold can be determined by measuring eye movement response while increasing the spatial frequency of the grating.

Similar to the clinical technique OKR paradigms have been adopted in many animal models including mice, rats,[12] chicken,[13] goldfish,[14] and zebrafish[14–21] for vision research. Zebrafish has become an important animal model in the field of neuroscience and ophthalmology.[22,23] The advantage of employing zebrafish as a model organism in eye research lies in the large number of offspring, a short generation time and the rapid development of its visual system. The OKR is already present in zebrafish larvae at 3 days past fertilization (dpf) and by 4 dpf it has almost reached the adult-like OKR behavior.[14,24,25] One previous study determined VA in zebrafish larvae at approximately 0.16 cyc/deg by measuring the gain of OKR (i.e., slow phase eye velocity/stimulus velocity) while altering the spatial frequency of the stimuli. In the same report, however, a considerably higher theoretical VA derived from the actual photoreceptor spacing was calculated as 0.24 cyc/deg.[18]

In the current study, we applied the OKR paradigm with a staircase approach to determine the threshold frequency in zebrafish larvae. A staircase procedure is defined by a repeatedly increasing and decreasing stimulus in predefined steps around the threshold.[26] This procedure generally allows reducing the influence of preceding steps and thus helps narrow down the uncertainty of the detected threshold and has been applied in measuring VA and contrast sensitivity in adult fish.[26] Our aim was to establish an easy and reliable method to measure VA in larval zebrafish, which should allow for a wide application, among others in modeling various human diseases that may be accompanied by impaired vision.

Materials and Methods

All experiments were performed in accordance with the animal welfare guidelines of the Federal Veterinary Office of Switzerland (FVO). Experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Animal Maintenance and Breeding

WIK (WIK) and Tuebingen (TU) wild-type zebrafish lines were bred and maintained as previously described.[27] Briefly, embryos were raised under a 14-hour light, 10-hour dark cycle in 28°C E3 Medium (in mM: 5 NaCl, 0.17 KCl, 0.33 CaCl2, and 0.33 MgSO4).[28] Larva at 5 to 6 dpf were used for the experiments.

Recording of Eye/Body Movement

Single larva were embedded in a transparent 21-mm diameter plastic tube filled with 3.5% methylcellulose. The tube was placed in the middle of a glass cylinder covered with a translucent screen and was illuminated from below by infrared (IR) emitting diodes. Eye movements were recorded by an IR sensitive charge couple device camera with a sample rate of 40 frames/second. The region of interest was manually defined. Throughout all experiments, both eyes were recorded disregarding the stimulation condition. Frames were processed by a custom-made eye recognition software written in Labview (National Instruments, Austin, Texas, USA).[29]

The Optokinetic Response (OKR)

Optokinetic response is a reflexive eye movement evoked by whole-field movements of the visual scene. The response consists of slow eye movements (slow phases) that follow the moving scene and saccades (fast phases) that are directed in the opposite direction. The OKR stimuli were composed of horizontally moving black/white vertical gratings and were projected by four beamers on a cylinder with the screen.[29] Stimulus properties such as grating velocity, grating contrast, and spatial frequency of the gratings were controlled by a custom-made program written in Labview (National Instruments, Austin, TX, USA). Spatial frequency is a measure of how often a predefined component periodically repeats per unit of a predefined visual space. In our analysis SPV was calculated by first removing all the saccades from the eye movement trace and afterwards by using a moving window that calculated the velocity within that period of time. The median value of these measurements is presented in the paper as "SPV."

Stimulus Conditions and Correct Response Criteria

We used an OKR paradigm to estimate the visual acuity (VA) in zebrafish larvae. Instead of calculating OKR slow phase eye velocity, we measured the directions of saccades during optokinetic stimulation to determine the responsiveness of the OKR. Our criteria for a correct OKR response was defined as four or more consecutive saccades in the opposite direction of the stimulus direction within one minute. The OKR stimulus properties applied were 10 dps absolute angular velocity (± for counterclockwise [ccw]/clockwise [cw] direction), 100% contrast, and a maximum illumination of 30.1 lux. Specifically, the "standard condition" was defined by a spatial frequency of 0.056 cyc/deg and the "below threshold condition" by a spatial frequency of 0.556 cyc/deg. Theoretically, all healthy 5 to 6 dpf larvae should exhibit OKR under the "standard condition." The "below threshold condition," on the other hand, should not evoke any OKR according to the retinal spacing of zebrafish larva at this developmental stage.[18]

Exclusion Criteria used in Selection of Subjects

All larva were preselected based on the following two conditions before any experiment and only selected larva were included as our experimental subjects. Larva with four or more consecutive saccades in either one of the two stimulus directions under the "below threshold condition" were excluded. Larva that failed to exhibit correct OKR response under the "standard condition" were also excluded.

Experimental Procedure 1: Visual Acuity

Visual acuity was estimated in individual larva by the measured highest spatial frequency threshold that could last elicit the OKR during binocular stimulation. A single recording consisted of 1-minute cw and 1-minute ccw moving stimulus. Each recording was repeated twice per step in the staircase procedure. In order to count a step as correct, a correct response had to occur in at least one of the two directions. The spatial frequency was increased stepwise from 0.056 cyc/deg by a step size of 0.028 cyc/deg until the cessation of correct responses. Returning to the spatial frequency of the last responsive step, the spatial frequency was increased again in smaller steps of 0.0056 cyc/deg until correct responses ceased and the spatial frequency threshold was reached (i.e., the spatial frequency of the last responsive step) for the first time. After cessation, the spatial frequency was in turn decreased in steps of 0.0056 cyc/deg until a correct response reoccurred.

Investigative Ophthalmology & Visual Science

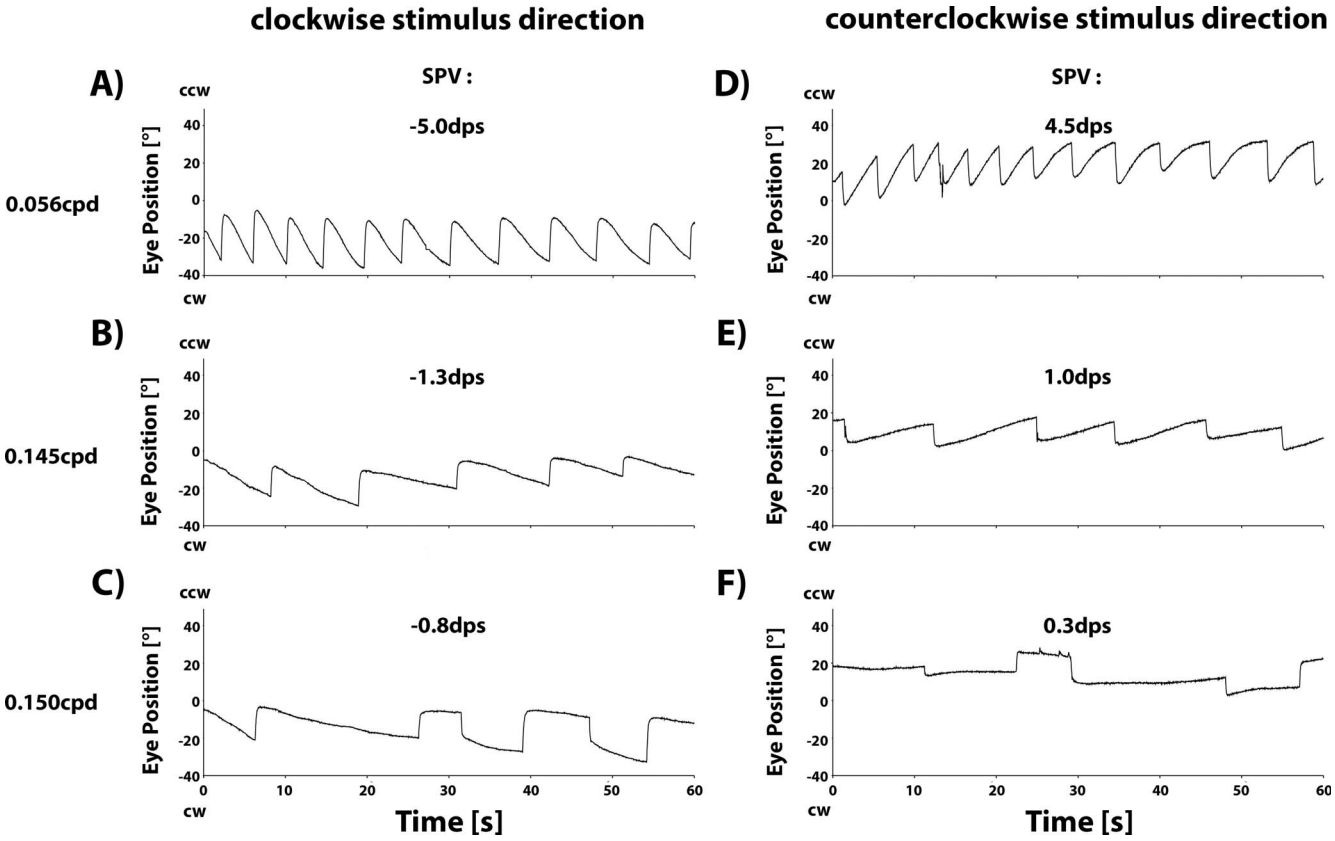

FIGURE 1. Sample OKR eye position traces under different stimulus conditions (**A**–**F**). (**A**, **D**) display typical OKR eye position traces under the standard condition. The SPV is −5.0 and 4.5 dps, respectively. The quick phase eye movements (saccades) are in the opposite direction of the stimulus direction. (**B**, **E**) display the eye traces at the threshold. Both the SPV and the amount of saccades decrease. The SPV is −1.3 dps and 1.0 dps, respectively. (**C**, **F**) display the eye traces one step below the threshold. The SPV further decreases to −0.8 dps and 0.3 dps, respectively. Note saccade movements below the threshold happen in both directions under a single stimulus direction.

Afterward, the spatial frequency was again increased in steps of 0.0056 cyc/deg until the spatial frequency threshold was reached for the second time. Similarly, after repeating the decreasing and then increasing of the spatial frequency in the same step size the spatial frequency threshold was reached for the third time. The median of these three threshold values is presented as the spatial frequency threshold. Twenty wild-type larvae (TU) randomly chosen from different clutches were used.

Experimental Procedure 2: Directional Difference

The experimental procedure was the same as in experimental procedure 1 except that experiments were performed separately for each stimulus direction. A single recording consisted of 1-minute recording in one direction. Each recording was repeated twice per step in the staircase procedure and a correct response had to occur in both recordings in order to proceed to the next step. Twenty wild-type larvae (WIK) randomly chosen from different clutches were used.

Experimental Procedure 3: Visual Acuity During Monocular Stimulation

In order to compare VA under monocular and binocular stimulation, 12 wild-type larva (TU) randomly chosen from different clutches were tested three times with a randomized order of left eye, right eye, and binocular stimulation. In each single trial, the experiment was carried out according to the experimental procedure 2. Monocular stimulation was achieved by covering 50% of the plastic tube with black tape.

RESULTS

Visual acuity is defined as the ability to distinguish two points at a given visual angle. We estimated the VA in 5 to 6 dpf wild-type zebrafish larvae based on the detected spatial frequency threshold of the OKR. In our experiments, we applied horizontally moving sine-wave, black-white vertical gratings as the visual stimuli for zebrafish larva. The OKR stimulus properties applied in all experiments were 10 degree per second (dps) absolute angular velocity (± for ccw/cw direction), 100% contrast, and a maximum illumination of 30.1 lux. Further, the "standard condition" was defined by a spatial frequency of 0.056 cyc/deg and the "below threshold condition" by a spatial frequency of 0.556 cyc/deg. Typical eye movement traces are shown in Figure 1. Unlike previous studies we determined VA by assessing saccade pattern instead of slow phase velocity (SPV). Our criteria for a correct OKR response was defined as four or more consecutive saccades in the opposite direction of the stimulus direction within 1 minute.

In the first experiment, we used a staircase approach to determine the spatial frequency threshold of the OKR based on the saccade criterion in 20 TU wild-type zebrafish larvae. An average spatial frequency threshold of 0.179 ± 0.013 cyc/deg

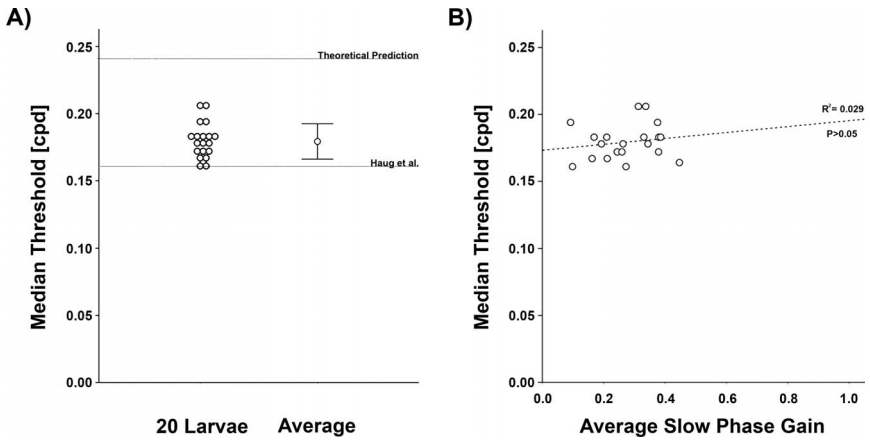

FIGURE 2. Median spatial frequency threshold and the correlation between the spatial frequency threshold and the OKR slow phase gain. Each data point represents the median spatial frequency threshold of a single subject. The average median threshold is 0.179 ± 0.013 cpd (**A**). Statistical analysis reveals no significant correlation between the median spatial frequency threshold and the average median peak slow phase gain (**B**). WIK wild-type larvae, $N = 20$.

(Fig. 2A). This result corresponded to 75% of the previously reported theoretical VA (0.24 cyc/deg) based on the retinal histology in zebrafish larvae.[18]

Unlike previous studies, we used saccade pattern instead of SPV as our response criterion. Previous studies determined VA by assessing the spatial frequency at which eye movement SPV could lastly be distinguished from spontaneous eye movements at 0.2 cyc/deg. In our study, the average median peak OKR SPV was measured as 4.881 ± 1.200 dps in the same 20 subjects under the standard stimulus properties. However, no significant correlation was found between VA and the average median peak SPV at standard condition (Pearson correlation –0.06, $P > 0.05$; Fig. 2B). The average median peak SPV at threshold spatial frequency was 1.936 ± 0.544 dps. There was also no significant correlation between VA and the average median peak SPV at the threshold (Pearson correlation –0.40, $P > 0.05$).

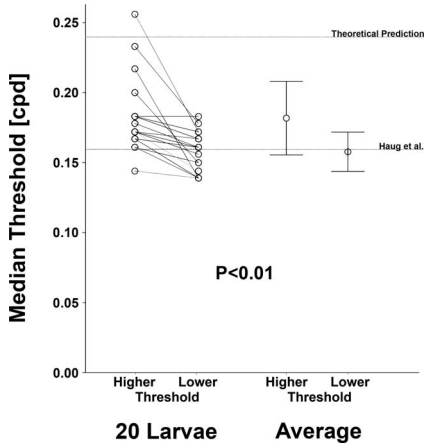

FIGURE 3. Ordered thresholds in both directions. Each subject gives a pair of spatial frequency thresholds in each of the two stimulus directions. All but one subject showed significantly different spatial frequency thresholds depending on the stimulus direction. In the *left part* of the figure the thresholds are ordered into "higher thresholds" and "lower thresholds." A paired *t*-test reveals significant difference between the two groups. In the *right part* of the figure the average median higher threshold (0.181 ± 0.026 cyc/deg) and the average median lower threshold (0.158 ± 0.014 cyc/deg) are depicted and can be compared with the theoretical spatial frequency threshold derived from the retinal photoreceptor spacing in zebrafish larvae (0.24 cpd, *upper dotted line*[18]) and the experimental value calculated based on eye velocity (0.16 cpd, *lower dotted line*[18]; TU wild-type larvae, $N = 20$).

Furthermore, we observed that most larva showed a directional difference in their optokinetic behavior around the threshold; in other words, they only showed residual responsiveness in one of the two stimulus directions (cw, ccw) when reaching the spatial frequency threshold. In a total of 20 larva, 11 showed stronger responsiveness to the cw stimulus direction, eight to the ccw direction, and only one larva did not show such directional-biased responsiveness.

To study this directional difference of the optokinetic responsiveness in individual larva, in the second experiment we measured the spatial frequency threshold in each stimulus direction independently. In a total of 20 WIK wild-type larvae, the average spatial frequency threshold of both directions was 0.170 ± 0.017 cyc/deg, which was not significantly different from the value measured in the first experiment ($P > 0.05$). However, most fish showed different responsiveness results in the two independent tests of the two directions: 12 of 20 larva showed a higher threshold in cw direction, while seven showed a higher threshold in ccw direction. One fish showed no difference between the two directions. For every fish we grouped the two measured thresholds into higher thresholds and lower thresholds disregarding the stimulus direction. The average of higher thresholds was 0.181 ± 0.026 cyc/deg, while the average of lower thresholds was 0.158 ± 0.014 cyc/deg (Fig. 3). A paired *t*-test revealed a highly significant difference between the higher and lower thresholds ($P < 0.01$).

The average median peak SPV at standard condition was 5.589 ± 1.303 dps. Ten of 20 larva showed a higher median peak SPV in cw stimulus direction, while the other 10 showed a higher median peak SPV in ccw stimulus direction. Similarly, we grouped the average median peak SPV into a higher SPV and a lower SPV group disregarding the stimulus direction. The average higher median peak SPV was 5.775 ± 1.395 dps, while the average lower median peak SPV was 4.783 ± 1.436 dps. Similar to the VA, a highly significant difference between the average median peak SPV in the two directions was found ($P < 0.001$). However, the average median peak SPV and threshold did not show a significant correlation ($P > 0.05$). Moreover, there was no significant correlation found between the stimulus direction of higher/lower average median peak SPV and the stimulus direction of higher/lower threshold (Fisher's exact test, $P = 1.000$, Table).

Table. Fisher's Exact Test for Correlation Between the Higher Median Peak SPV and Higher Threshold

	Higher Median Peak SPV	
Higher Threshold	**cw**	**ccw**
cw	6	6
ccw	4	3

The Fisher's exact test revealed no significant correlation between the direction of higher OKR gain and the direction of higher threshold spatial frequency. The numbers are the amount of subjects with the respective outcomes.

To further study the directional difference found under binocular stimulation and their correlation to right/left eye, we decided to evaluate the difference in VA under monocular and binocular viewing conditions in the third experiment. Thus, we measured the VA of a total of 12 TU wild-type larva under a randomized order of three stimulus conditions: left eye, right eye, and both eyes. The average spatial frequency threshold of both directions during binocular stimulation was 0.159 ± 0.015 cyc/deg. The average spatial frequency threshold during monocular stimulation of left eyes was 0.129 ± 0.008 and 0.128 ± 0.012 of right eyes. The VA under binocular viewing condition was significantly higher than during both monocular conditions ($P < 0.001$). However, no significant difference was found between the two monocular VA thresholds ($P = 0.829$). Next for every fish we grouped the three measured thresholds according to the order of the trials. The average median spatial frequency threshold of first trials was 0.139 ± 0.022, of second trials was 0.137 ± 0.015, and of third trials was 0.139 ± 0.021, which were not significantly different (ANOVA, $P = 0.95$).

Furthermore, similar to the VA test under binocular stimulation, in single fish under monocular stimulation we grouped the two measured thresholds into higher thresholds and lower thresholds disregarding the stimulus direction and found in both left and right eyes significant difference between the higher and lower thresholds (left eye: average higher/lower thresholds were 0.139 ± 0.011 / 0.118 ± 0.007 cyc/deg, $P < 0.001$ of a *t*-test; right eye: average higher/lower thresholds were 0.136 ± 0.015 / 0.119 ± 0.014 cyc/deg, $P = 0.008$ of a *t*-test). Moreover, among the 12 subjects, five were measured with a higher median VA from the right eyes and five had higher VA from left eyes (2 subjects were unbiased).

Moreover, we grouped the VA values by stimulus direction and by eye (left/right). For the left eye, the average VA during monocular stimulation from nasal to temporal was 0.135 ± 0.017 and 0.122 ± 0.007 cyc/deg during monocular stimulation from temporal to nasal ($P = 0.044$). Out of 12 larva, nine had higher VA values during stimulation from nasal to temporal and three had higher VA values during stimulation from temporal to nasal. For the right eye, the average VA during monocular stimulation from nasal to temporal was 0.129 ± 0.016 and 0.127 ± 0.017 cyc/deg during monocular stimulation from temporal to nasal ($P = 0.782$). Out of 12 larvae, seven had higher VA values during stimulation from nasal to temporal, four had higher VA values during stimulation from temporal to nasal, and one had equal values disregarding the stimulus direction.

Comparing both eyes, out of 12 larva, five had higher values during stimulation from nasal to temporal of either eye, five larva only had higher VA values during stimulation from nasal to temporal for one eye, and one larva had higher values during stimulation from temporal to nasal of either eye. Further only 3 out 12 larva had consistently higher VA values during stimulation moving in the same direction (e.g., ccw: nasal to temporal for the left eye/temporal to nasal for right eye and cw: temporal to nasal for the left eye/nasal to temporal for the right eye).

Further we decided to closer describe the SPVs found depending on spatial frequency and eye. At standard condition we found median peak SPVs of –5.968 ± 2.518 dps during cw binocular stimulation, 5.296 ± 2.004 dps during ccw binocular stimulation, –5.101 ± 1.125 dps during cw monocular stimulation of the left eye, 4.035 ± 1.230 dps during ccw stimulation of the left eye, –4.029 ± 1.044 dps during cw stimulation of the right eye and 4.797 ± 1.070 dps during ccw monocular stimulation of the right eye. At below threshold condition the peak SPVs were –0.27 ± 1.190 dps during cw binocular stimulation, 0.320 ± 1.095 dps during ccw binocular stimulation, –0.270 ± 1.169 dps during cw monocular stimulation of the left eye, 0.971 ± 1.032 dps during ccw stimulation of the left eye, 0.378 ± 1.475 dps during cw stimulation of the right eye, and 0.081 ± 1.420 dps during ccw monocular stimulation of the right eye. Pooled we found a peak SPV of 0.242 ± 1.259 dps with a range between –2.696 dps up to 2.344 dps during below threshold stimulation. At the threshold we found peak SPV of –2.564 ± 8.839 dps during cw binocular stimulation, 2.790 ± 0.881 dps during ccw binocular stimulation, –3.170 ± 0.763 dps during cw monocular stimulation of the left eye, 1.191 ± 0.833 dps during ccw stimulation of the left eye, –2.371 ± 0.419 dps during cw stimulation of the right eye, and 2.806 ± 0.278 dps during ccw monocular stimulation of the right eye. Pooled we found a peak SPV of –0.099 ± 2.735 dps with a range from –4.291 dps up to 4.471 dps. Of these values found at threshold, 47.223% fall into the range found in below threshold condition. There was again no correlation between SPV (at both below threshold and standard condition) and threshold.

Discussion

We estimated the VA in 5 to 6 dpf zebrafish larva by eliciting the OKR and determining their spatial frequency threshold. Sample eye traces from one representative larva are shown in Figure 1. Figures 1A and 1D show typical eye movement traces at the spatial frequency of 0.056 cyc/deg. Under the standard stimulus condition (defined by a spatial frequency of 0.056 cyc/deg), the subject showed no saccades in an incorrect direction and had an average SPV of –5.0 dps in the cw direction and 4.5 dps in the ccw direction. At the spatial frequency threshold we could still measure the correctly directed saccades however the SPV dropped to –1.3 dps in the cw direction and 1.0 dps in the ccw direction (Figs. 1B, 1E). Under the below threshold condition (defined by a spatial frequency of 0.556 cyc/deg; Figs. 1C, 1F), the saccades were seen in random directions regardless of the stimuli and the SPV further decreased to –0.8 and 0.3 dps, respectively. Comparing the eye traces between at threshold and below threshold conditions (Figs. 1B, 1E versus Figs. 1C, 1F), in almost 50% of all cases the low SPV was in the same range of SPVs found in spontaneous eye drifts. On the other hand, one could clearly and easily distinguish a response from a nonresponse by the saccade directions comparing with the stimuli. As a consequence, instead of calculating the eye velocity we looked at eye position traces and recorded the saccade direction and saccade numbers as our measures to determine the OKR responsiveness.

In a previous report using the SPV as a measure to determine the VA in zebrafish larva, the spatial frequency threshold was reported to be around 0.16 cyc/deg.[18] As a comparison in the current study, a clear OKR response could be seen at considerably higher spatial frequencies up to 0.24 cyc/deg, which is as high as the theoretical value (0.24 cyc/

deg) based on the retinal morphology in zebrafish larva.[18] We shall mention that in some situations certain residual responsiveness could still be observed below the spatial frequency threshold, even though the SPV was no longer detectible. Thus, the VA reported in the present study was still likely underestimated. This may be accounted for by our relative strict correct response criteria in order to minimize the false positive response. Furthermore, the influence of the eye movement habituation on the responsiveness to the stimuli might well be more pronounced near the stimulus threshold. Other factors that could explain for the considerably lower experimental VA (average 0.179 cyc/deg) to the theoretical VA (0.24 cyc/deg) are the decreasing alertness of the subjects during the long testing procedure, the immature visual system in 5 to 6 dpf larva, and certain limitations of the experimental setup. These include the animal restraining methods (e.g., using methylcellulose, which certainly decreases the best visibility of stimulus and can to some degree also restrict eye movements), stimulus properties (such as brightness), raw data quality (noise), and the algorithm used to filter noise.

Based on our results it is advisable not to use the SPV as a measure for determining the VA: around the threshold the OKR SPV is normally as low as below 1.5 dps, which may not be distinguished from the spontaneous eye drifts or any recording noise. Thus, depending on the resolution of the recoding setup and the reliability of the analysis software, one could easily underestimate the VA to a certain, or in some case to a considerably big, degree. Another disadvantage of using the SPV as a measure would be the difficulty to compare VA with small differences among subjects. Moreover, we found neither a correlation between the spatial frequency threshold and the SPV under standard OKR condition (Fig. 2B) nor a correlation between the spatial frequency threshold and the SPV at the threshold (data not shown).

Our results imply a distinct difference between OKR responsiveness (e.g., how well the eye sees the moving gratings) and actual OKR eye movements (e.g., how fast the eye follows the moving scene) in zebrafish larvae as the measured VA can be inconsistent with the SPV. This result is not difficult to explain: while VA is mostly related to the retina morphology, slow phase eye movements involve continuous excitation of the whole ocular motor system including the muscular function, and other neuronal structures which may easily be exhausted and do not relate to vision per se.

When larva were tested separately under two different stimulus directions binocularly, the majority of the subjects showed a significantly higher spatial frequency threshold in one of the two directions (Fig. 3). Change in alertness as a cause of the directional bias is excluded, as the stimulus always started with a random direction and no correlation could be found between first direction and higher threshold (data not shown). One possible explanation for the difference may be associated with the asymmetrical early development of the optical system in 5 to 6 dpf zebrafish larva. Hence, in order to measure the absolute VA one should take into account such directional difference when applying OKR in a VA testing paradigm in zebrafish larva. One should also take into account that VA during monocular stimulation is significantly lower than during binocular stimulation. This was also shown to be true for some adult zebrafish and could be due to the missing binocular field or due to the reduced visual input, and therefore reduced OKR trigger.

During monocular stimulation, approximately two-thirds of the subjects had a higher VA threshold when stimulus moved from nasal-to-temporal (N-T), although *t*-test revealed only significant difference for one eye, this is in strike contrast to the characteristic asymmetry of the lower OKR gain of the N-T motion that is generally observed in lateral-eyed vertebrates.[15] Note in our current study we compared the OKR responsiveness based on saccade direction instead of slow phase velocity or gain. Thus, one likely explanation for such inconsistency could be, again, one behavioral bias is originated from the retina (viewing) while the other involves the entire ocular motor system (tracking). For the retina it might be less critical to "see" a T-N moving target as it likely stays in the small binocular visual field or shift to the monocular visual field of the other eye; however, for the ocular motor system to "track" the N-T motion may be undesirable as animals have adapted to such moving scene whenever moving forward.[30]

Instead of using the optokinetic SPV as a measure for determining the VA in zebrafish larva, we developed a new VA testing paradigm, in which we apply the staircase method and determined the optokinetic responsiveness based on eye position traces and the saccadic eye behaviors. This way we not only avoided wrongly comparing the VA with the SPV or OKR gain, because our study has proved no correlation between these values, but also enhanced the detecting sensitivity, regardless of the low signal-to-noise ratio around the threshold. Further it also provides a measuring method that gives consistent VA values with only simple analysis needed.

Acknowledgments

The authors thank Marco Penner for his excellent technical support, Daniel Rappoport for the fruitful discussions, Stephan Neuhauss and Kara Dannenhauer for providing fish materials and for fish care.

This work was supported by the Swiss National Science Foundation (SNF) Grants PMPDP3_139754 (Marie Heim-Vögtlin programme; Bern, Switzerland) and 31003A-118069, the Betty and David Koetser Foundation for Brain Research (Zurich, Switzerland), and the Dr. Dabbous-Foundation (Zurich, Switzerland).

Disclosure: **S.Y. Bögli**, None; **M. Afthinos**, None; **G. Bertolini**, None; **D. Straumann**, None; **M.Y.-Y. Huang**, None

References

1. Sloan LL. Measurement of visual acuity; a critical review. *AMA Arch Ophthalmol*. 1951;45:704–725.
2. Westheimer G. Visual acuity. *Ann Rev Psychol*. 1965;16:359–380.
3. Wall M, Johnson CA. Principles and techniques of the examination of the visual sensory system. In: Miller NR, Newman NJ, Biousse V, Kerrison JB, eds. *Walsh and Hoyt's Clinical Neuro-Ophthalmology*. Philadelphia, PA: Lippincott Williams & Wilkins; 2005;89–93.
4. Beck RW, Cleary PA, Anderson MM Jr, et al. A randomized, controlled trial of corticosteroids in the treatment of acute optic neuritis. The Optic Neuritis Study Group. *N Engl J Med*. 1992;326:581–588.
5. Rowe FJ, Walker M, Rockliffe J, et al. Care provision for poststroke visual impairment. *J Stroke Cerebrovasc Dis*. 2015; 24:1131–1144.
6. Hyon JY, Yeo HE, Seo JM, Lee IB, Lee JH, Hwang JM. Objective measurement of distance visual acuity determined by computerized optokinetic nystagmus test. *Invest Ophthalmol Vis Sci*. 2010;51:752–757.
7. Han SB, Han ER, Hyon JY, Seo JM, Lee JH, Hwang JM. Measurement of distance objective visual acuity with the computerized optokinetic nystagmus test in patients with ocular diseases. *Graefes Arch Clin Exp Ophthalmol*. 2011;249: 1379–1385.
8. Teller DY. The forced-choice preferential looking procedure: a psychophysical technique for use with human infants. *Infant Behav Dev*. 1997;2:135–153.

9. Dobson V, Teller DY. Visual acuity in human infants: a review and comparison of behavioral and electrophysiological studies. *Vision Res*. 1978;18:1469–1483.
10. Dayton GO Jr, Jones MH, Aiu P, Rawson RA, Steele B, Rose M. Developmental study of coordinated eye movements in the human infant. I. Visual acuity in the newborn human: a study based on induced optokinetic nystagmus recorded by electro-oculography. *Arch Ophthalmol*. 1964;71:865–870.
11. David NJ. Optokinetic nystagmus. A clinical review. *J Clin Neuroophthalmol*. 1989;9:258–266.
12. Prusky GT, West PW, Douglas RM. Behavioral assessment of visual acuity in mice and rats. *Vision Res*. 2000;40:2201–2209.
13. Schmid KL, Wildsoet CF. Assessment of visual acuity and contrast sensitivity in the chick using an optokinetic nystagmus paradigm. *Vision Res*. 1998;38:2629–2634.
14. Beck JC, Gilland E, Tank DW, Baker R. Quantifying the ontogeny of optokinetic and vestibuloocular behaviors in zebrafish, medaka, and goldfish. *J Neurophysiol*. 2004;92:3546–3561.
15. Huang YY, Neuhauss SC. The optokinetic response in zebrafish and its applications. *Front Biosci*. 2008;13:1899–1916.
16. Rinner O, Rick JM, Neuhauss SC. Contrast sensitivity, spatial and temporal tuning of the larval zebrafish optokinetic response. *Invest Ophthalmol Vis Sci*. 2005;46:137–142.
17. Tappeiner C, Gerber S, Enzmann V, Balmer J, Jazwinska A, Tschopp M. Visual acuity and contrast sensitivity of adult zebrafish. *Front Zool*. 2012;9:10.
18. Haug MF, Biehlmaier O, Mueller KP, Neuhauss SC. Visual acuity in larval zebrafish: behavior and histology. *Front Zool*. 2010;7:8.
19. Goldsmith P, Harris WA. The zebrafish as a tool for understanding the biology of visual disorders. *Semin Cell Dev Biol*. 2003;14:11–18.
20. Brockerhoff SE, Dowling JE, Hurley JB. Zebrafish retinal mutants. *Vision Res*. 1998;38:1335–1339.
21. Risner ML, Lemerise E, Vukmanic EV, Moore A. Behavioral spectral sensitivity of the zebrafish (Danio rerio). *Vision Res*. 2006;46:2625–2635.
22. Bilotta J, Saszik S. The zebrafish as a model visual system. *Int J Dev Neurosci*. 2001;19:621–629.
23. Fadool JM, Dowling JE. Zebrafish: a model system for the study of eye genetics. *Prog Retin Eye Res*. 2008;27:89–110.
24. Easter SS, Nicola GN. The development of vision in the zebrafish (Danio rerio). *Dev Biol*. 1996;180:646–663.
25. Easter SS, Nicola GN. The development of eye movements in the zebrafish (Danio rerio). *Dev Psychobiol*. 1997;31:267–276.
26. Treutwein B. Adaptive psychophysical procedures. *Vision Res*. 1995;35:2503–2522.
27. Mullins MC, Hammerschmidt M, Haffter P, Nüsslein-Volhard C. Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. *Curr Biol*. 1994;4:189–202.
28. Haffter P, Granato M, Brand M, et al. The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development*. 1996;123:1–36.
29. Chen CC, Bockisch CJ, Bertolini G, et al. Velocity storage mechanism in zebrafish larvae. *J Physiol*. 2014;592:203–214.
30. Collewijn H, Noorduin H. Conjugate and disjunctive optokinetic eye movements in the rabbit, evoked by rotatory and translatory motion. *Pflugers Arch*. 1972;335:173–185.

Investigative Ophthalmology & Visual Science